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A high prevalence *CCDC103* p.His154Pro mutation causing primary ciliary dyskinesia disrupts protein oligomerisation and is associated with normal diagnostic investigations

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What is the key question?

Can gene sequencing improve diagnosis of the inherited respiratory condition primary ciliary dyskinesia in patients with unclear clinical diagnostic investigations?

What is the bottom line?

CCDC103 p.His154Pro missense mutations cause up to 20% PCD cases in UK South Asian populations but diagnosis can be difficult in this group using standard clinical diagnostic tests because results are often normal; we therefore propose that genetic analysis is an essential part of the diagnostic algorithm to complement standard clinical tests to improve diagnostic accuracy.

Why read on?

Patients with primary ciliary dyskinesia may be missed using current pathology-based diagnostic protocols therefore genetic screening can provide valuable support in obtaining a definitive diagnosis.

For Twitter: 140 character conclusion:

Diagnosis can be difficult in PCD with *CCDC103* p.His154Pro mutations and genetic testing is essential in the high-risk UK South Asian community.

ABSTRACT

Rationale: Primary ciliary dyskinesia is a genetically heterogeneous condition characterised by progressive lung disease arising from abnormal cilia function. Approximately half of patients have situs inversus. The estimated prevalence of primary ciliary dyskinesia in the UK South Asian population is 1:2,265. Early, accurate diagnosis is key to implementing appropriate management but clinical diagnostic tests can be equivocal.

Objectives: To determine the importance of genetic screening for primary ciliary dyskinesia in a UK South Asian population with a typical clinical phenotype, where standard testing is inconclusive.

Methods: Next-generation sequencing was used to screen 86 South Asian patients who had a clinical history consistent with primary ciliary dyskinesia. The effect of a *CCDC103* p.His154Pro missense variant compared to other dynein arm-associated gene mutations on diagnostic/phenotypic variability was tested. *CCDC103* p.His154Pro variant pathogenicity was assessed by oligomerisation assay.

Results: Sixteen of 86 (19%) patients carried a homozygous *CCDC103* p.His154Pro mutation which was found to disrupt protein oligomerisation. Variable diagnostic test results were obtained including normal nasal nitric oxide levels, normal ciliary beat pattern and frequency and a spectrum of partial and normal dynein arm retention. Fifteen (94%) patients or their sibling(s) had situs inversus suggesting *CCDC103* p.His154Pro patients without situs inversus are missed.

Conclusions: The *CCDC103* p.His154Pro mutation is more prevalent than previously thought in the South Asian community and causes primary ciliary dyskinesia that can be difficult to diagnose using pathology-based clinical tests. Genetic testing is critical when there is a strong clinical phenotype with inconclusive standard diagnostic tests.

INTRODUCTION

Primary ciliary dyskinesia (PCD; OMIM: 244400) is an inherited disorder affecting motile cilia. Patients usually present with a history of neonatal respiratory distress and suffer from lifelong symptoms of chronic wet cough and rhinitis. Recurrent chest infections ultimately lead to bronchiectasis and a progressive decline in lung function.¹ Approximately half of patients have situs inversus and other situs abnormalities, due to ciliary dysmotility in the embryonic node.² Fertility can also be affected by defective cilia in the fallopian tubes and non-motile sperm tail flagella.

The estimated prevalence of PCD in the UK is 1:15,000, but as high as 1:2,265 in the UK South Asian population.^{1,3} Early diagnosis is important to maintain lung function, and appropriately treat symptoms to reduce morbidity and mortality.⁴ Diagnosis can be complex and requires a combination of tests for cilia functional and ultrastructural defects.^{5,6} PCD is caused by mutations in genes encoding proteins conferring structural stability to the cilia and governing ciliogenesis. It is genetically heterogeneous with >200 individual mutations in more than 30 genes known to cause PCD. To date these account for approximately 65% of cases.^{1,7-11} UK Genetic Testing Network approved tests are offered in two centres (http://www.labs.gosh.nhs.uk/media/764464/ciliopathies_v8.pdf).¹²

CCDC103 mutations were first reported, in 2012, in patients with dynein arm loss and a typical clinical PCD phenotype.¹³ *CCDC103* is an oligomeric coiled-coil domain protein that is found tightly bound to the ciliary axoneme where it is thought to help facilitate ciliary motility by participating in attachment of the dynein arms to the axoneme. The protein was found to stabilize cytoplasmic microtubules against cold depolymerization in an in vitro assay.¹³ A missense variant previously identified in *CCDC103* to cause a single amino acid

change to the protein, p.His154Pro (rs145457535) was previously described as a hypomorphic mutation since mutant p.His154Pro *CCDC103* induced intermediate partially rescued disease phenotypes when expressed in a zebrafish *CCDC103*-null model, suggesting some protein function was retained.¹³ In cilia from p.His154Pro-positive patients some cilia showed a partial dynein arm defect with the outer dynein arms (ODA) at least partially assembled, compared to loss-of-function *CCDC103* mutations causing complete ODA loss. In agreement with this, loss-of-function *CCDC103* mutation patients had largely static cilia, whilst patients carrying a homozygous p.His154Pro mutation exhibit a mixed (static and motile) ciliary beat pattern.¹³

We conducted genetic screening of 86 PCD patients of South Asian (mostly Pakistani) origin, detecting that a significant proportion (19%) were homozygous for *CCDC103* p.His154Pro variant. Amongst these, many were at high risk of being undiagnosed without genetic testing, due to normal diagnostic results obtained in PCD investigations. Using electron microscopy and protein biochemistry we have sought to further determine the pathogenic nature of the p.His154Pro mutation.

METHODS

Patient selection

Eighty-six patients of South Asian (primarily Pakistani) descent with clinical signs and symptoms of PCD were identified from the UK National PCD Diagnostic and Management Services at The Royal Brompton Hospital, London, University Hospital Southampton, Birmingham Children's Hospital, Bradford Royal Infirmary and Leicester General Infirmary.

1 14

Genetic screening

All participants gave written informed consent to take part in this study. The protocol was approved by the London Bloomsbury Research Ethics Committee (08/H0713/82). High throughput screening used next-generation sequencing, either whole exome sequencing (WES) or targeted gene panel sequencing. Sequencing and variant identification methods are published for whole exome sequencing^{12 15} or used custom gene panels (Illumina TruSeq Custom Amplicon or Agilent SureSelect Focused Exome (proprietary product) and SureSelectXT custom panel design systems) and a standardised variant calling pipeline.¹⁶ Sanger sequencing was used for variant confirmation and familial segregations. As shown in Table 1, of the 16 p.His154Pro cases, this mutation was detected by WES in case #1-6 and #9, by use of a 'clinical exome' commercial panel in cases #7 and #8 and in cases #10-16 by use of custom targeted gene panels containing the known PCD genes and other candidate PCD disease genes.

Comparator group

The comparator patient group consisted of 16 of the 86 individuals tested. This group was closely age and gender matched to the *CCDC103* p.His154Pro group. All had a dynein arm

defect on electron microscopy and all were proven negative for the *CCDC103* p.His154Pro mutation.

FEV1 measurements

Spirometry was performed according to American Thoracic Society/European Respiratory Society recommendations.¹⁷ Forced expiratory volume in 1 second (FEV1) z-scores were calculated using the Global Lungs Initiative parameters.¹⁸

Diagnostic tests

Screening and diagnostic testing was performed according to the PCD National Service protocols. Investigations included nNO, nasal brushing analysed by HSVI for ciliary beat frequency and pattern¹⁹ and quantitative electron microscopy for ciliary ultrastructure²³.

Additional detail on the method is provided in an online data supplement. When results were inconclusive or inconsistent, patients were offered repeat testing.

Protein biochemistry on recombinant CCDC103 protein

Site-directed mutagenesis (QuikChange kit, Agilent Technologies UK Ltd) was used to generate the p.His154Pro mutation in an N-terminal His10 tagged *H. sapiens CCDC103* cDNA subcloned into pET16b vector that was synthesized using *Escherichia coli* codon bias.

²⁰ Following transformation into *E. coli* BL21 (DE3), protein expression was induced by addition of 2 mM IPTG for two or more hours. Following sonication, His₁₀-tagged proteins were dissolved in 8 M urea, then very slowly refolded by their dilution into 1 litre of 20 mM Tris.Cl pH8.0 150 mM NaCl. Proteins were then purified by Ni²⁺-affinity chromatography as described previously, using 20 mM Tris pH 8.0, 500 mM NaCl, 250 mM imidazole for elution.

²⁰ Samples concentrated by ultrafiltration through Amicon Ultra-4 ultrafiltration units were subject to gel filtration in a calibrated Superose 6 10/300 column attached to an ÄktaPurifier-

10 chromatography workstation.²⁰ The mutant protein was very hard to make and only a little was able to be refolded, indeed even the precipitated material was clearly different to the wildtype protein being more "sticky". Hence, a lower concentration of the mutant protein is apparent in Figure 4. This experiment was done at one concentration (the highest we were able to achieve): for wildtype protein this was ~80 ug/ml and for the p.His154Pro protein this was ~30 ug/ml.

Statistical analysis

The *CCDC103* p.His154Pro and comparator patient groups were closely matched for age and gender. Data was not normally distributed and therefore groups were compared using non-parametric statistical tests. $P < 0.05$ was considered statistically significant.

RESULTS

A PCD-causing mutation *CCDC103* p.His154Pro is prevalent in UK individuals of South Asian origin with a clinical phenotype of PCD

Next generation sequencing analysis in 86 patients of South Asian descent with suspected PCD revealed 16 patients (19%) from 12 independent families homozygous for a previously published single base change (NM_001258395.1: c.461A>C) mutation in *CCDC103* that predicts the amino acid substitution p.His154Pro (Table 1). Consistent with their homozygous segregation pattern, all 16 patients were children of consanguineous parents and had dynein arm defects or normal ultrastructure.

Due to the high frequency of this mutation, we examined whole exome sequence data available from 1,542 unaffected parents with similar ethnic backgrounds participating in the Born-in-Bradford study, all of UK South Asian, primarily Pakistani heritage.²¹ This revealed six heterozygous carriers of the *CCDC103* p.His154Pro substitution (E. Sheridan, unpublished data). The ExAc database²² of exome sequencing results from 60,706 unrelated individuals free from paediatric disease records an allele frequency for p.His154Pro three times as high in 8,256 South Asian individuals (0.003) compared to 33,345 North Europeans (0.001). No p.His154Pro homozygote individuals were identified in the entire Born-in-Bradford or ExAc cohorts.

Amongst the sixteen *CCDC103* p.His154Pro homozygote PCD patients identified, normal diagnostic test results were apparent as highlighted in Table 1. Although PCD was strongly suspected in all cases, five of the sixteen patients (case 9 and 13-16 in Table 1) did not receive a definitive disease diagnosis until their genotype was confirmed. Remarkably in the case of patient 15, this individual had been deemed to not have PCD and was discharged from respiratory care. She was re-tested due to her situs inversus and the finding that her brother, who remained under clinical suspicion, had this *CCDC103* mutation. Thirteen of the 16 *CCDC103* p.His154Pro patients (81%) have situs inversus, including in two families the presence of two siblings with situs inversus, and no families without situs inversus. Situs inversus is reported to affect approximately 50% patients with PCD with dynein arm defects² and affected 56% of the PCD comparator group in this study (Table 2). 70% of the comparator group had situs inversus in their family amongst affected individuals.

FEV₁ measurements showed that impact on lung function was similar in the group of 16 p.His154Pro patients compared to the PCD comparator group (Table 1, Figure 1). The

comparator group of 16 South Asian individuals with PCD all had dynein arm deficiency, but due to different genetic causes since this group comprised three *DNAAF1*,²³ two *DNAAF3*,²⁴ two *DNAH5*,²⁵ three *LRRC6*²⁶ and two *ZMYND10*²⁷ cases whilst four were genetically undefined. The mean difference for clinical measures between the *CCDC103* p.His154Pro and comparator groups is presented in Table 2, showing an equivalent age and gender composition. Individuals from both groups had symptoms typical of PCD as detailed in Supplementary Table 1. There was a higher rate of glue ear and positive sputum microbiology and a lower rate of bronchiectasis in the *CCDC103* p.His154Pro group compared to controls, but due to the small number of patients and heterogeneous nature of PCD it is difficult to interpret the significance of these findings.

PCD caused by the *CCDC103* p.His154Pro mutation can be associated with normal nasal nitric oxide (nNO) results

We proceeded to retrospectively analyse clinical phenotypes of the sixteen affected South Asian patients carrying the homozygous p.His154Pro missense mutation in more detail, as presented in Table 1. Of the 16 p.His154Pro patients tested, seven (43%) had normal nNO levels, above 77nl/min which is a recommended diagnostic cut off.²⁸ Of these 7 patients, most (patients 5, 7, 11, 12 and 13) were subject to repeat measurements at subsequent clinic appointments. Patient 12 displayed persistent values within the normal range up to 5 years from the first measurement. Mean nNO was significantly higher than the low levels consistently detected in the comparator PCD patient group (Table 2).

PCD caused by the *CCDC103* p.His154Pro mutation can be associated with areas of normal ciliary beat frequency on high speed video light microscopy

High-speed video microscopy revealed that amongst the 16 homozygous p.His154Pro individuals, nine (56%) had ciliary beat frequencies within the normal range of 8.5-16.8Hz (mean 11.6Hz) (Table 1). The other seven p.His154Pro patients (patients 1-6 and 13) had a reduced beat frequency with a mean of 3.9Hz (range 0-7.7Hz), which is more in keeping with typical results found for PCD patients and more specifically found for *CCDC103* patients expressing loss-of-function alleles.^{13 29} The comparator group of PCD patients displayed a reduced ciliary beat frequency with a mean of 1.3Hz (Table 2), which is similar to the reported reduced ciliary beating seen in published PCD cases associated with other causes of dynein arm loss. In half the p.His154Pro patients the beat pattern of cilia was completely or largely normal in some strips of epithelium (patients 5-7, 9, 10, 12, 15, 16, Table 1) whilst static, slow or dyskinetic in others. Some His154Pro patients (7, 10, 16) demonstrated a full beat pattern and almost immotile cilia together within the same sample (Supplementary videos 1-3). Therefore the motility in p.His154Pro individuals was variable, compared to the fully motile cilia of healthy controls (Supplementary video 4) and the completely static cilia seen in patients from the comparator group (Supplementary video 5).

PCD caused by the *CCDC103* p.His154Pro mutation can be associated with normal ultrastructural appearance by electron microscopy

Seven of the 16 *CCDC103* p.His154Pro individuals (44%) had a defect of the ciliary inner and outer dynein arms demonstrated by transmission electron microscopy (cases 1-7 in Table 1) The other nine p.His154Pro individuals (cases 8-16) had TEM that either showed an absence of the inner dynein arm, or that was considered normal or inconclusive despite extensive interrogation. The spectrum of ultrastructural defects found in p.His154Pro

individuals is illustrated with representative examples in Figure 2. Overall, the inner dynein arm appeared to be the most affected structure in these cases, with significant retention of outer dynein arms. Quantification of these dynein arm defects is shown in Figure 3. Published normal range counts from >200 non-PCD respiratory controls are also shown for comparison (Figure 3, grey box). Notably, samples from four *CCDC103* p.His154Pro patients were completely within this normal range, a further two were closer to the normal range than the diagnostic range and two although not quantifiable due to small numbers were also reported as normal. When a partial defect of the outer dynein arm was seen, an assessment of proximity to the epithelial cell surface - as judged by the presence of neighboring microvilli - showed that ODA loss typically occurred at the distal end of the cilia, towards the tips.

A summary shown in Table 2 highlights that the majority of *CCDC103* p.His154Pro cases clearly differed from the non-*CCDC103* comparator group, which have a near complete absence of both dynein arms. This is highly significant in terms of outer dynein arm loss ($p < 0.05$), with only 27% ODA absence in the *CCDC103* p.His154Pro cilia compared to 89% in the comparator group which carry mutations in other dynein arm-loss associated PCD genes. Inner dynein arms were also clearly more retained in *CCDC103* p.His154Pro cilia, but this difference did not reach statistical significance. The overall relative lack of disturbance to dynein arm structures means that *CCDC103* p.His154Pro TEM overlaps with that of non PCD controls.

Biochemical analysis of the *CCDC103* p.His154Pro mutation reveals an abrogation of its oligomerisation capacity

We conducted gel filtration of purified CCDC103 p.His154Pro protein and compared this to the normal protein using previously established methods,²⁰ to assess the functional viability of the mutant form of the protein. The p.His154Pro variant appears to be a highly disruptive mutation since the ability of CCDC103 to oligomerize is significantly disrupted by the mutation, as shown in the chromatogram (Figure 4). The lack of oligomers that the wildtype protein forms (~250 kDa) is however accompanied by a large increase in the void volume of the mutant protein. We speculate that this material which is >2 MDa, is aggregated protein, rather than organized oligomers.

DISCUSSION

We report from genetic screening of a PCD cohort ascertained through UK National PCD Services that the *CCDC103* c.461A>C; p.His154Pro mutation accounts for disease in approximately one fifth of affected individuals in the highly consanguineous South Asian PCD community, associated with loss of both the outer and inner dynein arms of cilia. Therefore, a significant risk of disease arises from the presence of this important frequent mutation which is typically carried in homozygous state, one copy inherited from each parent. Direct screening for this specific p.His154Pro mutation in South Asian PCD cases could be an economical diagnostic approach.

Despite the relatively high prevalence of *CCDC103* p.His154Pro, this mutation is not the sole cause of the increased incidence of PCD reported in the British South Asian community.³ Our screen also revealed patients in the group of 86 screened that carry causal mutations in a number of other genes including *CCDC40*, *DNAAF1*, *DNAAF3*, *HEATR2*, *LRRC6*,

ZMYND10 and *RSPH4A*. The *CCDC103* p.His154Pro defect is not exclusive to the UK Asian community and has also been detected in a patient from an Irish travelling family ³⁰ as well as in two North European origin families with PCD from our studies (unpublished data), all of whom have dynein arms defects.

This study highlights extensive variability in the diagnostic results for patients carrying biallelic *CCDC103* p.His154Pro mutations. Normal standard diagnostic test results have led to *CCDC103* p.His154Pro patients being discharged from the PCD clinic, and in some cases the diagnosis of PCD in a *CCDC103* p.His154Pro patient was only finally confirmed following the genetic test result. Fifteen (94%) patients had situs inversus suggesting *CCDC103* p.His154Pro patients without situs inversus may be missed either due to lack of referral or due to normal diagnostic tests.

The homozygous p.His154Pro positive patients represent an expanded phenotype for PCD, since without genetic results many in this group may not have been considered to meet the current UK clinical diagnostic criteria for PCD. This would lead to uncertainty for the patient and their parents and may result in unnecessary further investigation into the cause of their symptoms. One South Asian patient in the comparator group had a TEM phenotype similar to that of the *CCDC103* p.His154Pro group. This individual also had a nNO level of >77nl/min, but a screen for the *CCDC103* mutation was negative (Figure 3). This case, along with other patients in the UK national diagnostic clinics, suggests that there will be other mutations which cause PCD with normal diagnostic results. It is difficult to explain the variability in the nasal NO. This variability is temporal within a patient and seen even between siblings. We speculate that this may be a broader reflection of ciliary function in the sinuses such that NO levels may only be low in *CCDC103* p.His154Pro patients when cilia are static (maybe during

an infection or other external insult) and may normalise with improved ciliary function. The possible reasons why levels might be low in PCD were recently summarized.³¹

Interestingly, preservation of some cilia structure and motility in *CCDC103* p.His154Pro patients is not apparently accompanied by significantly preserved lung function, since the FEV₁ range in p.His154Pro patients is equivalent to the comparator group and respiratory capacity is equally reduced in these individuals. Another missense mutation causing PCD, *ZMYND10* p.Val16Gly, was similarly found to cause a mixed cilia beat defect with a significant degree of retained cilia motility but this is also not yet associated with milder disease course.²⁷ In this study, larger numbers of *CCDC103* p.His154Pro patients and controls should be analysed to confirm this observation and genotype – phenotype relationships could be further investigated with more sensitive tests for staging lung disease such as Lung Clearance Index, CT scan or radiolabeling methods screening mucociliary clearance rates.³²

³³ Patients with the *CCDC103* p.His154Pro mutation represent an interesting cohort for targeted pharmacogenetic therapies due to their prevalence and since the dynein components required for normal ciliary beating appear to still be present in the patient's cilia, albeit at variable levels.

The finding of nNO levels within the normal range in *CCDC103* p.His154Pro patients highlights the importance of considering the full clinical history in conjunction with nNO testing in patients with suspected PCD. Clinicians should proceed to further testing in cases with a high index of clinical suspicion, using the NO test as part of a multidisciplinary diagnostic protocol rather than a stand-alone screening test. This is not the first report of normal nNO results in patients with PCD. Some patients with *RSPH1* mutations are also

reported to have levels of NO close to normal and we expect further such cases to be reported as the complex genetic landscape of PCD diagnosis is unraveled further.³⁴

The apparently normal/mixed cilia beat pattern found in half of p.His154Pro cases was notably high, but this finding is supported by previous work indicating that this represents a hypomorphic allele in cilia function tests.¹³ Awareness of these cases is critical when assessing diagnostic samples in the laboratory, if the observer inadvertently tends to select beating strips for analysis over those that are static when scanning the sample at 5-20 times magnification before full analysis with high speed video, thereby inadvertently missing this defect when a significant portion of the sample has a normal co-ordinated beat. This places an emphasis on diagnostic centres to increase awareness and expertise of the operators when assessing nasal biopsies by high-speed video microscopy. HSVM does not quantify the power of the ciliary beating, but in cases with a normal stroke and frequency there could be weakness in the strength of the beating that might not be detected by any of the current diagnostic tests.

In this study, TEM diagnosis often revealed a pattern of intermittent IDA and ODA loss in *CCDC103* p.His154Pro patients which was distinct from cases of dynein arm absence due to mutations in other dynein-loss associated PCD genes e.g. *LRRC6*, *DNAAF1* and *ZYMND10*. The distinction was most significant for ODA retention with *CCDC103* p.His154Pro cases, with comparatively high levels of preserved ODAs seen in patient's cilia whilst the IDAs were more often missing. Despite analysis of >100 cilia cross sections, usually sufficient for diagnosis, the loss of dynein was not always detected by electron microscopy. However, it has long been accepted that normal ultrastructure by electron microscopy cannot exclude PCD.³⁵

³⁶ Our study appears to confirm in *CCDC103* p.His154Pro patients the previous evidence that

CCDC103 mutations confer a loss of distal ODAs containing DNAH9, not ODAs in the proximal half of the cilia closer to the epithelial surface.¹³

This study contributes strong evidence that *CCDC103* p.His154Pro is pathogenic rather than a benign polymorphism. It is present at very low frequency in the non-PCD population and no *CCDC103* p.His154Pro homozygote individuals were detected in large scale screening of 9,798 South Asian and 33,345 North European controls. Also, segregation analysis performed where parental samples were available in the affected families studied here showed an inheritance pattern fully consistent with recessive disease (Table 1). The CCDC103 protein remains poorly characterised but previous studies show that it usually forms dimers and higher order oligomers. It is thought to help in generating a high-affinity site on the doublets for outer arm assembly, either through direct interactions or indirectly, by modifying the underlying microtubule lattice.²⁰ The oligomerization capacity of CCDC103 is a property of the central region of the protein (R.P.K. and S.M.K., unpublished observations) which contains a highly conserved RPAP3_C domain that spans residues 96-189 of the protein and is predicted to function in protein-protein interactions.²⁰ The His154 amino acid is located within an alpha helix of this RPAP3_C functional domain and substitution of the cyclic side chain of proline at this position would enforce a conformation predicted incompatible with alpha helical secondary structure. Thus, altering His154 is expected to disrupt the protein's secondary structure and consequently its function. Our current results reveal that the His154Pro mutant form retains the ability to dimerize but shows little oligomer formation, suggesting that this property is disrupted by the mutation and that CCDC103 may have two distinct self-interaction domains. We hypothesise that gene mutations causing instability or depletion of CCDC103 protein may make the attachment of the dynein arms more susceptible to physical, infective or inflammatory insult. We cannot determine if the presence of normal

cilia in our patients is temporal or spatial however, as results appear to vary from one biopsy to the next e.g. in TEM data for case 11, 12, 15 and 16 in Table 1; cell culture and repeating investigations may be useful in these cases.

It can often be difficult to make or exclude a diagnosis of PCD due to poor sensitivity of electron microscopy and genetic testing and the poor specificity of nasal nitric oxide measurement.³⁷ Currently, diagnosis using multiple tests is recommended. These tests are often complex and require specialist equipment and skills to interpret. Consequently, several patients continue to have an indeterminate diagnosis. Awareness of variants such as *CCDC103* p.His154Pro may allow targeted gene screening in patients with an indeterminate diagnosis.

In conclusion, the *CCDC103* p.His154Pro variant is prevalent in the UK South Asian community and likely to be found in South Asian patients worldwide. This patient group should therefore undergo genetic testing for c.461A>C, especially if (partial) dynein arm absence is suspected. These patients frequently present a diagnostic dilemma, due to inconclusive results of multiple clinical diagnostic tests. This study expands the diagnostic phenotype which we consider to be PCD, since in some cases pathology-based tests can be equivocal as the cilia can beat at least partially in a co-ordinated manner, at the correct speed and may appear structurally normal. PCD is widely understood to be an underdiagnosed condition and this appears to be the case for *CCDC103* p.His154Pro patients, who demonstrate a high level of situs inversus probably indicative of a lack of recognition making their diagnosis liable to be missed.³⁸ We anticipate that studies such as this, in combination with easier access to high throughput and economically achievable genetic screening, should greatly increase disease recognition and understanding. We have highlighted the importance

of multidisciplinary testing, repeat testing and genotyping in patients with a highly suggestive history for PCD.

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Demographics					Symptoms			Diagnostic test results					
ID	Sex	Age (yrs)	Age diag (yrs)	Family History	FEV ₁ z-score	Main Symptoms	Situs	Nasal NO (nl/min)	CBF (Hz)	Ciliary beat pattern	Electron microscopy (1st brushing)	Electron microscopy (repeat brushing)	Genetics: sequencing protocol and segregations
1	M	7	7	No	-3.45	Chronic cough, developmental delay	SI	ND	0	Immotile	IDA +ODA	IDA +ODA	CM Watson et al. 2014
2	M	4	1	Sib 3	-2.25	Nasal discharge	SI	1	0.95	Mostly immotile with occasional residual movement	IDA +ODA	IDA +ODA	CM Watson et al. 2014
3	M	12	10	Sib 2	-2.75	Neonatal respiratory distress, nasal discharge	SS	11	2.9	Mostly immotile with occasional residual movement	IDA +ODA	IDA +ODA	CM Watson et al. 2014
4	M	13	<1	No	ND	Neonatal respiratory distress, Gilbert syndrome, epilepsy	SI	7	5.9	Mostly immotile with occasional residual movement	IDA +ODA	IDA +ODA	CM Watson et al. 2014
5	M	13	6	Sib 6	-1.52	Recurrent chest infections	SI	87	5.79	Normal ciliary beat pattern reduced frequency	IDA +ODA	IDA +ODA	WES ^P
6	F	15	4	Sib 5	-0.61	Recurrent chest infections, Eustachian tube dysfunction, nasal discharge	SS	43	7.66	Normal ciliary beat pattern reduced frequency	IDA +ODA	IDA +ODA	WES ^P
7	F	18	9	No	-1.58	Recurrent chest infections, nasal discharge, bilateral glue ear	SI	111	10.6	Mixed Sample 1: normal ciliary beat pattern sample 2: Immotile	IDA +ODA	IDA +ODA	Illumina TruSeq custom gene panel ^M
8	M	9	6	No	-0.08	Wet cough, recurrent chest infections, conductive hearing loss	SI	57	8.8	Dyskinesia	IDA	IDA	Agilent SureSelect Focused Exome ^P
9	F	7	7	CHD	0.43	Chronic cough	SI	33	10.9	Normal ciliary beat pattern	Normal	ND	CM Watson et al. 2014
10	M	10	<1	Sib 11 & 12	-1.32	Neonatal respiratory distress, nasal discharge	SI	26	11.5	Mixed: Normal areas, immotile areas, dyskinetic areas	Normal	Normal	Illumina TruSeq custom gene panel ^B
11	F	21	10	Sib 10 & 12	ND	Recurrent chest infections, asthma, persistent collapse of right lower lobe	SI	293	8.5	Mixed: Mostly reduced forward and recovery stroke	IDA	Normal	Illumina TruSeq custom gene panel ^B
12	M	13	<1	Sib 10 & 11	-1.24	Neonatal respiratory distress, nasal discharge	SS	214	16.3	Normal ciliary beat pattern	IDA	Normal	Illumina TruSeq custom gene panel ^B
13	F	29	29	No	ND	Bronchiectasis, infertility	SI	239	4.31	Mixed: Mostly reduced forward and recovery stroke. Static patches	Normal	Insufficient	Agilent SureSelectXT ^B
14	F	1	1	No	ND	Neonatal respiratory distress, nasal discharge, wet cough	SI	ND	16.76	Dyskinesia	Normal	Inconclusive	Agilent SureSelectXT
15	F	21	21	Sib 16	ND	Recurrent chest infections in childhood. No ear, chest or nasal	SI	151	10.9	Normal ciliary beat pattern	IDA	Normal	Agilent SureSelectXT ^M
16	M	18	18	Sib 15	ND	Sensory neural hearing impairment, recurrent chest infections, chronic nasal congestion and rhinitis	SI	276	9.9	Mixed. 80% normal ciliary beat pattern. 20% immotile cilia on strips.	IDA	Normal	Agilent SureSelectXT Ciliome_651 ^M

Table 1. Clinical history of *CCDC103* p.His154Pro cases summarising diagnostic investigations Normal test results are highlighted in bold. Age diag, age diagnosis confirmed; SI, situs inversus; SS, situs solitus; NO, nitric oxide; CBF, ciliary beat frequency; IDA, inner dynein arm; ODA, outer dynein arm; ND, not done. Segregation analysis indicated by M (mother is carrier), P (father is carrier) or B (both parents carriers), unaffected siblings have not been tested.

Clinical features	N	<i>CCDC103</i> p.His154Pro	N	Comparator group
Age (mean SD)	16	11.8 (4.8)	16	11.9 (8.8)
Gender (% Male)	16	67%	16	63%
Nasal Nitric Oxide (nl/min)	14	63 (63)	9	12 (7)**
Ciliary beat frequency (Hz)	16	7.5 (4.7)	16	1.3 (3.6)**
% Cross sections with IDA absent on TEM	13	45% (24)	16	63% (30)
% Cross sections with ODA absent on TEM	13	19% (12)	16	89% (20)**
FEV ¹ z score (median IQR)	10	-1.4 (-0.8, -2.1)	13	-1.8 (-1.5, -2.1)
Situs Inversus	16	81%	16	56%

Table 2. Comparison of mean values of clinical tests obtain in *CCDC103* p.His154Pro cases versus a comparator group The *CCDC103* p.His154Pro cases are described in Table 1 and the comparator group are South Asian origin *CCDC103* p.His154Pro-negative cases with a confirmed absent dynein arms defect and genetic results as described in the main text. We only show TEM data for patients in whom more than 100 cilia were counted. Data shown as the mean, with standard deviation shown in brackets (StDev) unless otherwise stated. ** p<0.005.

FIGURE LEGENDS

Figure 1. Comparison of predicted FEV1 in *CCDC103* p.His154Pro cases versus a comparator group

The *CCDC103* p.His154Pro cases are described in Table 1 and the comparator group are South Asian origin *CCDC103* p.His154Pro-negative cases with a confirmed absent dynein arms defect. Mutations carried by the comparator group are in *DNAAF1* (3 cases), *DNAAF3* (2 cases), *DNAH5* (2 cases), *LRRC6* (3 cases), *ZMYND10* (2 cases), whilst 4 cases were genetically undefined.

Figure 2. Transmission electron microscopy of *CCDC103* p.His154Pro patients

Representative cilia cross sections from *CCDC103* p.His154Pro patients show within the same micrograph (A) absent outer and partially absent inner dynein arms and (B) presence of outer dynein arms only and presence of both inner and outer dynein arms. (C) Inset shows presence of outer but not inner dynein arms. Black arrows indicate example outer dynein arms. White arrows indicate inner dynein arms. Scale bar, 100 nm.

Figure 3. Quantitative transmission electron microscopy survey of inner and outer dynein arm loss in *CCDC103* p.His154Pro patients versus a comparator group

By surveying >100 cross sections in each patient sample we performed quantitative electron microscopy to determine the percentage of arm defects in cilia from individuals homozygous for the hypomorphic p.His154Pro *CCDC103* mutation. Note that quantitative plots have only been included within this graph if more than 100 cilia were counted and that all data has been collected at a single centre to ensure uniform results; data collected for one sample from a separate centre was therefore excluded. Red diamonds and triangles indicate results from 16 *CCDC103* p.His154Pro homozygote PCD patients, where triangles indicates the result of 4

repeat nasal brushings performed on patients marked by a diamond. The comparator group of 16 individuals with PCD, indicated by other symbols as shown, consists of 3 cases with *DNAAF1* mutations (open diamonds), 2 *DNAAF3* (black squares), 2 *DNAH5* (dark blue diamonds), 3 *LRRC6* (2 as light blue diamonds, 1 contained within the filled purple circle), 2 *ZMYND10* (contained within the filled purple circle) and 4 cases in whom no mutations in known genes could be identified (grey squares). Six *CCDC103* p.His154Pro samples (27%) of p.His154Pro samples showed complete lack of both outer and inner dynein arms comparable to other gene mutations in the graph (purple circle). The grey shaded area represents normal range counts from >200 non PCD respiratory controls. Four *CCDC103* p.His154Pro patients had counts within this normal range (one is a repeat sample (triangle) which showed similar data). Individuals with *CCDC103* p.His154Pro mutation have a trend towards a distinctive pattern of partial loss of dynein arms that diverges from total dynein arm loss in the comparator group.

Figure 4. CCDC103 p.His154Pro oligomerisation capacity Chromatograms of wildtype (red trace) and His154Pro (green trace) CCDC103 native proteins separated in a calibrated Superose 6 10/300 gel filtration column. The data is plotted as absorbance at 280 nm (in mAU) against elution volume (ml). Both proteins show strong dimer peaks at ~60 kDa. However, only the wildtype form generates a series of higher-order oligomers with an approximate mass of ~250 kDa. Aggregated material (>2 MDa) eluted in the void volume.

SUPPLEMENTARY VIDEOS

Supplementary video 1

HSVM of p.His154Pro homozygote patient 10 captures fully beating cilia

Supplementary video 2

HSVM of p.His154Pro homozygote patient 10 captures mixed dyskinetic and faintly moving/immotile cilia

Supplementary video 3

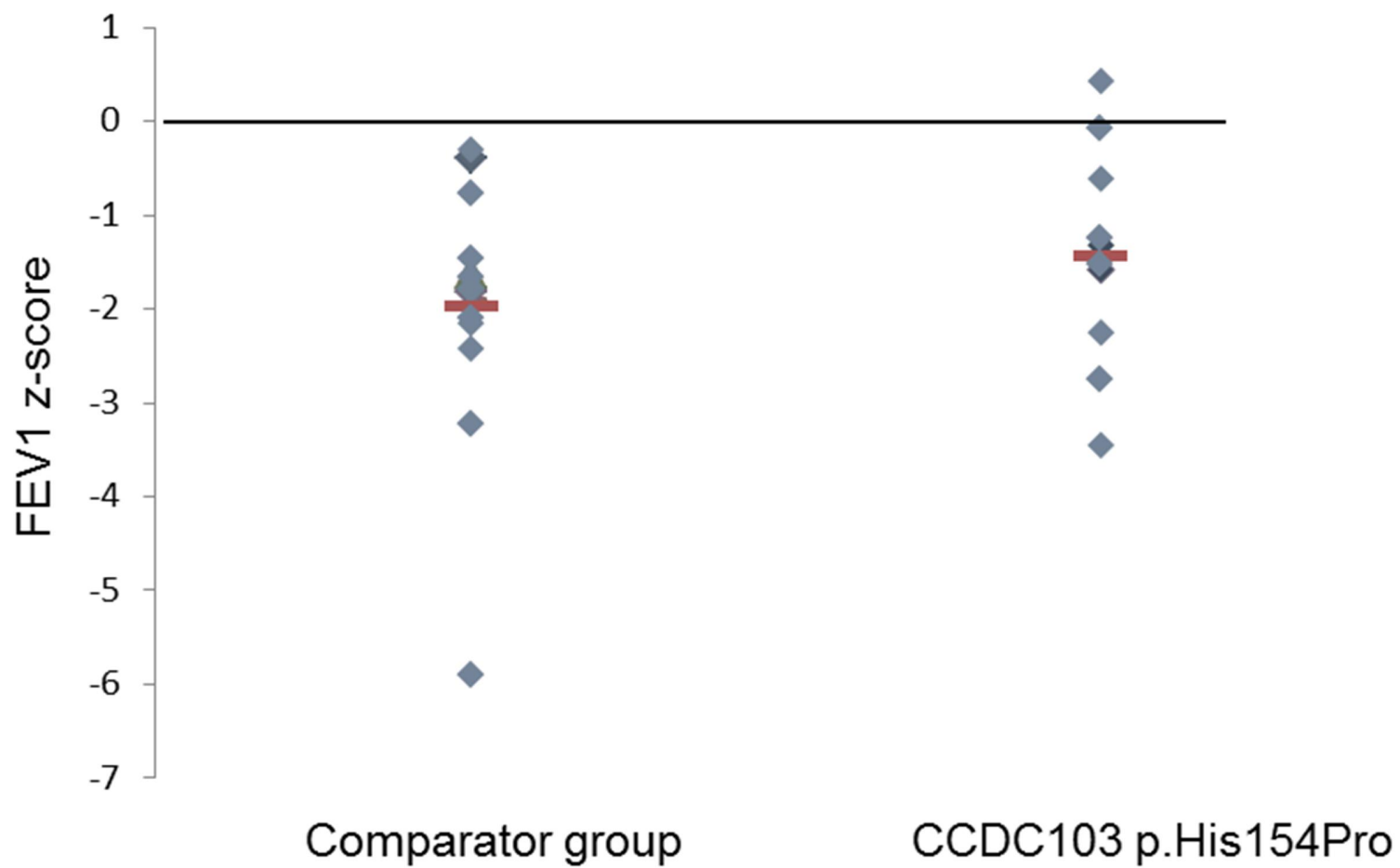
HSVM of p.His154Pro homozygote patient 16 captures mixed beat pattern (immotile and beating)

Supplementary video 4

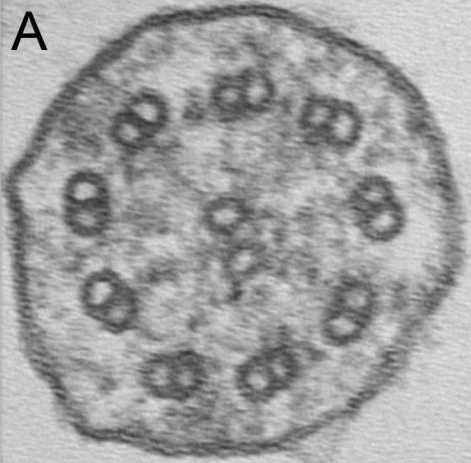
HSVM of patient carrying ZMYND10 mutations (static) from comparator group

Supplementary video 5

HSVM of health control (normal beat)



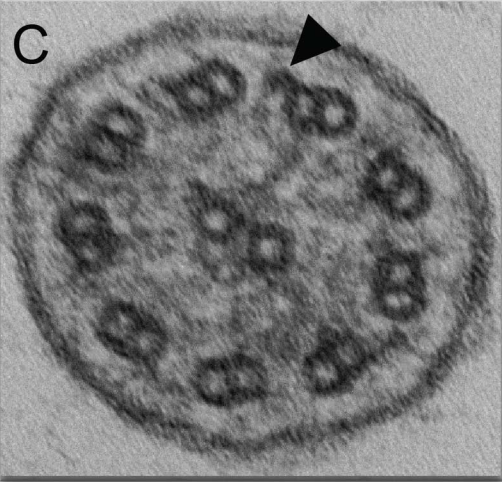
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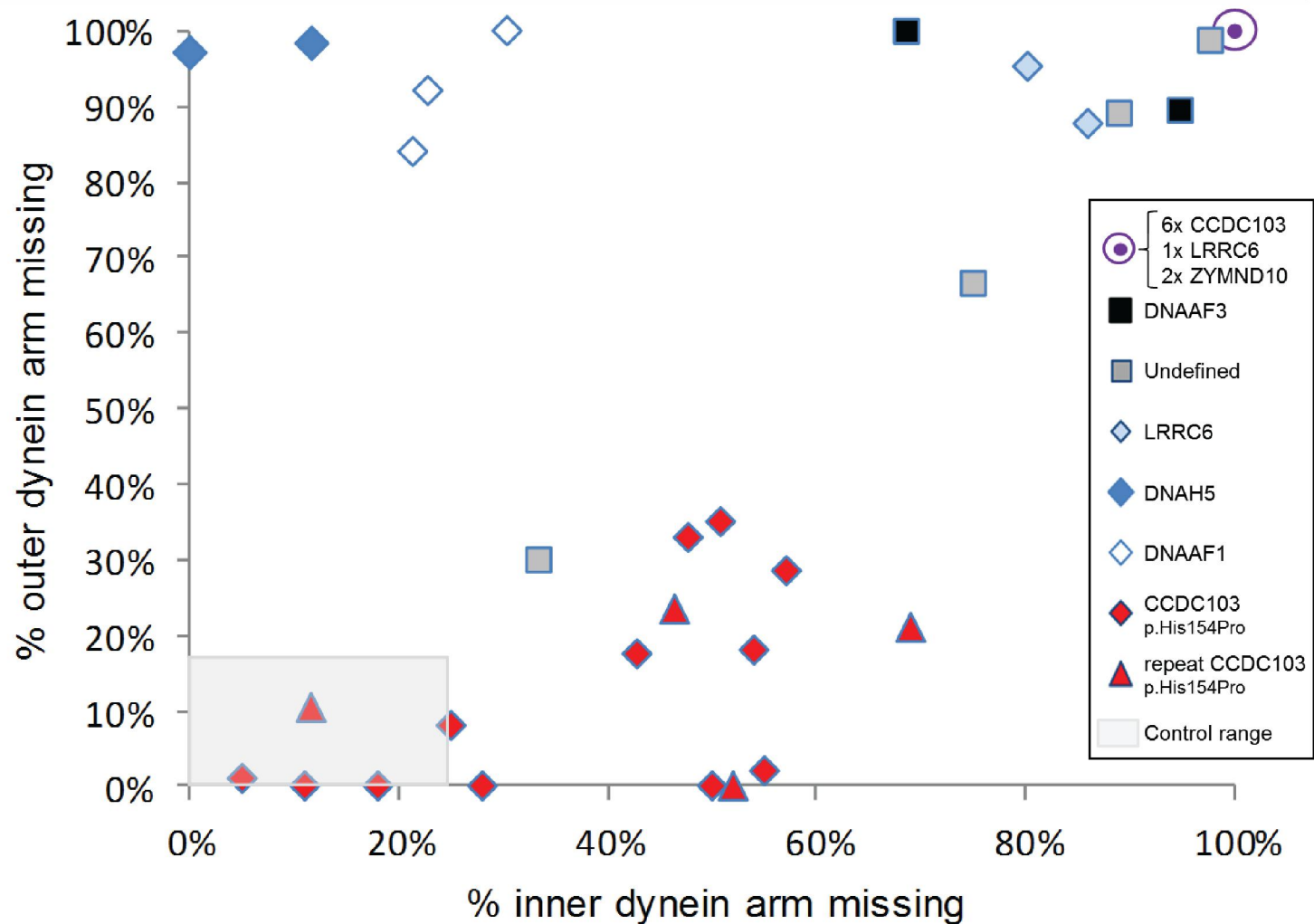


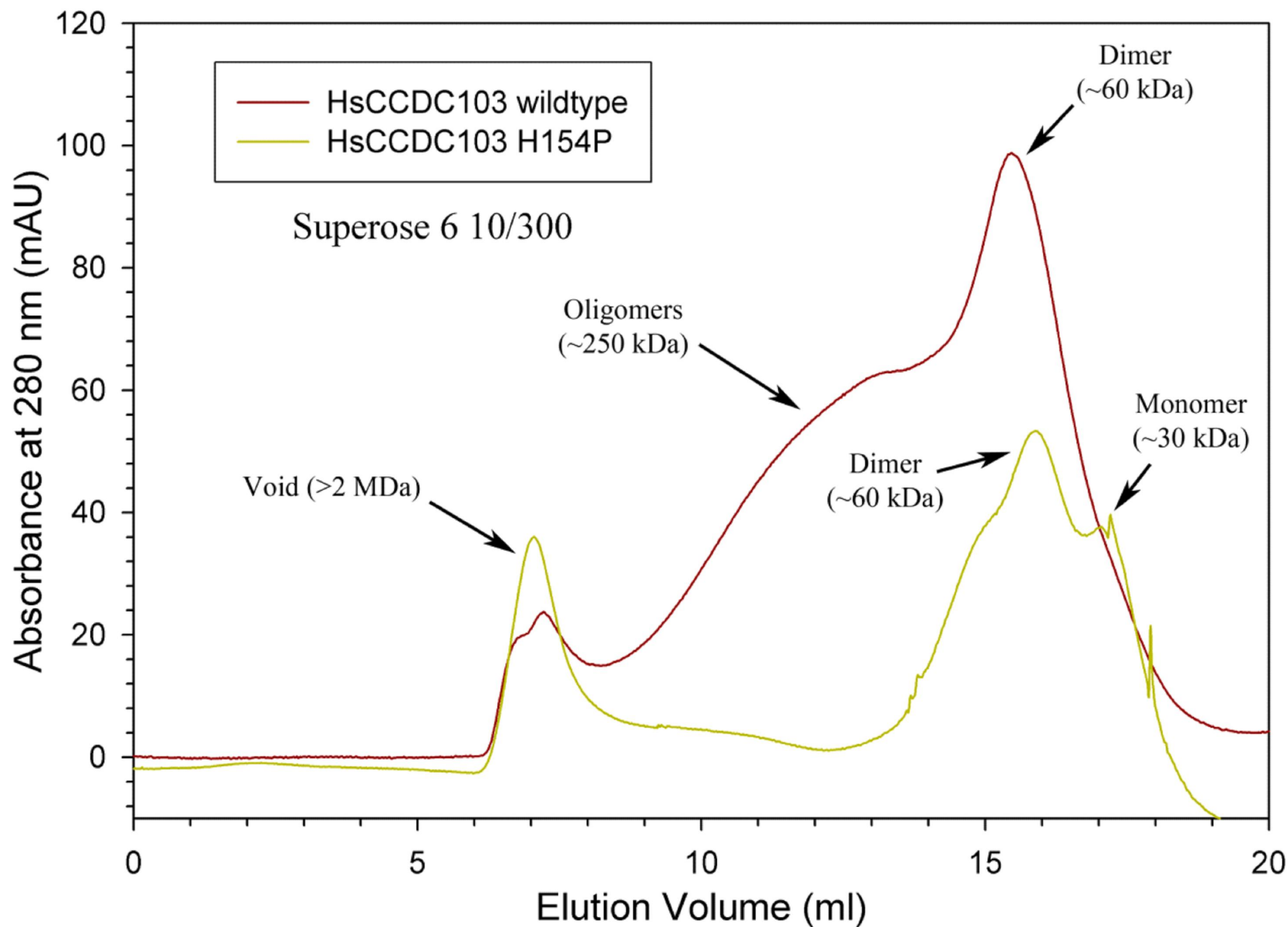
B



C







Online data supplement

Clinical features described at last review	N	CCDC103 p.His154Pro	N	Comparator group
Age (mean SD)	16	11.8 (4.8)	16	11.9 (8.8)
Gender (% Male)	16	67%	16	63%
History of neonatal respiratory distress	14	71%	13	77%
Wet Cough				
Nasal Symptoms	14	93%	15	100%
	13	85%	15	100%
Glue ear	10	70%	16	56%
Bronchiectasis on HRCT	8	50%	7	86%
Sputum microbiology*	14		15	
<i>Pseudomonas aeruginosa</i>		0%		7%
<i>Haemophilus influenza</i>		29%		7%
<i>Streptococcus pneumonia</i>		14%		7%
No growth/ oral flora		57%		87%

Supplementary Table 1. Comparison of clinical features between the *CCDC103* p.His154Pro mutation group of patients versus the South Asian age matched comparator group carrying other dynein arm-associated gene mutations. Data obtained from the most recent clinical review by the UK PCD management team, obtained via a retrospective review of clinical records. * Some individuals grew multiple organisms

Supplementary Methods

Diagnostic tests

Nasal Nitric Oxide

Nasal Nitric oxide testing using a breath hold maneuver was performed using one of 3 analyzers: a Logan LR2000 (Logan Research Ltd., UK) or a NIOX Mino or NIOX Flex (Aerocrine AB, Sweden). Results are reported in nl/min for standardization between analyzers (1).

Light microscopy

To biopsy the nose, strips of mucosa were scraped from the inferior turbinate with a cytology brush (2). The biopsy sample was placed in M199 maintenance medium at 37°C and examined by light microscopy to assess ciliary beat pattern and frequency. High speed video microscopy was performed using an oil immersion 100x lens on a minimum of 10 strips of epithelium. Beating cilia were recorded at 500 frames per second and played back at 60 frames per second to analyse cilia beat pattern and frequency (in Hz) of both top and side profiles (3).

Electron microscopy

Samples were fixed in 2.5% glutaraldehyde in cacodylate buffer and processed for electron microscopy. Defects were quantified using the method described by Shoemark et al 2012 (4). Briefly, cells were washed in sodium cacodylate buffer, post-fixed with 1% osmium tetroxide and centrifuged in agar or agarose to generate a pellet. Using a series of increasing concentrations of methanol followed by propylene oxide, cells were dehydrated before embedding in resin then 70-90nm sections were cut using an ultramicrotome, mounted onto copper grids. Heavy metal staining was with uranyl acetate and lead citrate. Assessment of the respiratory epithelium and ciliary ultrastructure were made on a transmission electron microscope. Quantification of cells, microtubular arrangement in the axoneme and presence of dynein arms was performed by a clinical electron microscopist blinded to the case information. Care was taken to assess cilia from a number of healthy cells from locations proximal and distal to the epithelial cell surface. Transverse sections of cilia were methodically quantified until either the entire section or 300 cilia had been counted.

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